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LC–MS/MS-analysis of prostaglandin E_2 and D_2 in microdialysis samples of rats

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Abstract

For the determination of prostaglandins in microdialysis samples, usually immunoassays are used. However, these assays may show cross-reactivity among various prostaglandins. To overcome this problem a specific method for the determination of prostaglandin E_2 and D_2 in rat microdialysis samples by using liquid chromatography–electrospay ionization-tandem mass spectrometry (LC–ESI-MS/MS) is described. Prostaglandin E_2 and D_2 were extracted from microdialysis samples with liquid–liquid extraction using deuterated prostaglandin D_2, [$^2H_4$]-PGD_2, as internal standard. Subsequently, prostaglandins were separated with a phenomenex Synergi Hydro-RP column and determined with a PE Sciex API 3000 mass spectrometer equipped with a turbo ion spray interface operating in negative ionization mode. The method showed a LLOQ of 25 pg/ml for prostaglandin E_2 and 50 pg/ml for prostaglandin D_2. The applicability of the method is shown in rat spinal cord microdialysis samples following peripheral nociceptive stimulation.

Keywords: PGE_2; PGD_2; Chromatography; Tandem mass spectrometry; ESI; Microdialysis

1. Introduction

Prostaglandins (PG) are important mediators of physiological and pathophysiological effects. On account of a certain stimulus arachidonic acid is released from the cell membrane into the cytoplasm and is then converted by cyclooxygenases, COX-1 and COX-2, to PGH_2 [1,2]. COX-1 is constitutively expressed in most tissues and is involved in homeostasis and mucosa protection, whereas COX-2 expression is upregulated in inflammatory processes.

Cyclooxygenases, also known as prostaglandin H synthases, functionally reflect enzymes with a cyclooxygenase and a peroxidase activity. The cyclooxygenase function converts arachidonic acid to the hydroperoxide PGG_2 which is then reduced with the peroxidase function to PGH_2. Following PGH_2 formation, various prostaglandin synthases transform PGH_2 to the four major prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 and to thromboxane A_2 (TXA_2). Prostaglandins are important mediators in inflammation, pain and fever. Especially PGE_2 is involved in spinal nociceptive processing [3]. In contrast to PGE_2, PGD_2 may be involved in anti-inflammatory processes [4,5]. An injection of formalin for instance into a rat hind paw leads to a rapid PGE_2 increase in the spinal cord within minutes which is mainly caused by constitutively expressed COX-1 [3,6,7]. In these experiments microdialysis was used to collect the samples and immunoassays to determine PGE_2 concentrations, but the role of PGD_2 is unclear. However, determination of PGD_2 concentrations in biological samples using the commercially available PGD_2-methoxime-immunoassays (PGD_2-MOX) is problematic. This immunoassay is based on the conversion of PGD_2 to a stable methoxime-derivative.

Microdialysis is a suitable method to collect samples from the spinal cord in freely moving animals over a period of time, but the subsequent immunological assays to monitor prostaglandin levels may be tricky. First, the rather small sample volume of approximately 75 μl and the usually low prostaglandin concentrations do not allow for the determination of more than one compound in one sample. Second, immununological cross-reactivity among prostanoids may

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occur. To overcome these disadvantages of immunoassays, gas chromatography combined with mass spectrometry may be used. Although GC–MS and GC–MS/MS show a higher sensitivity as compared to immunoassays, LC–MS and liquid chromatography–tandem mass spectrometry (LC–MS/MS), they require time-consuming sample derivatisation and long run times [8,9]. LC–MS/MS may be an adequate method to evaluate such microdialysis samples because of its sensitivity and selectivity and no need of derivatisation. We here describe an assay using LC–MS/MS to determine PGE_2 and PGD_2 among other prostanoids.

2. Experimental

2.1. Materials

Acetonitrile, methanol and water for chromatography were of HPLC grade and were obtained from Mallinckrodt Baker (Griesheim, Germany). Formic acid (p.a.) was purchased from Merck KgaA (Darmstadt, Germany). All prostaglandins used as standards were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Positions 3 and 4 of the internal standard [$^2H_4$]-PGD_2 were labeled with two deuterium atoms (Fig. 1).

Artificial cerebrospinal fluid (ACSF) consisted of 115.7 mM sodium chloride, 2.6 mM potassium chloride, 0.9 mM magnesium chloride hexahydrate, 2.1 mM sodiumbicarbonate, 2.5 mM disodiumhydrogenphosphate dihydrate, 1.3 mM calcium chloride dihydrate and 3.5 mM D-(+)-glucose. In order to adjust pH to 7.2 the solution was bubbled with 5% CO_2 in 95% O_2. ACSF chemicals and ethyl acetate were obtained from Fluka (Seelze, Germany) and were of p.A. quality.

Ketamine used for induction of anesthesia of animals was received from Pharmacia & Upjohn GmbH (Erlangen, Germany) and xylazine from Bayer AG (Leverkusen, Germany). Isoflurane was obtained from Abbott GmbH (Wiesbaden, Germany). Formalin was purchased from the local hospital pharmacy and was prepared freshly each time.

Solvent A was acetonitrile/water (40:60, v/v, pH 6.8), solvent B was methanol and solvent C was methanol/water/formic acid (25:75:0.1, v/v, pH 2.8). Acetonitrile/water/formic acid (40:60:0.01, v/v, pH 3.4) was used as solvent D and acetonitrile/water/formic acid (40:60:0.1, v/v, pH 2.8) as solvent E.

2.2. Instrumentation

Sample analysis was perfomed by using liquid chromatography–electrospay ionization-tandem mass spectrometry (LC–ESI-MS/MS). The HPLC equipment consisted of an HPLC pump (Jasco PU-1585, Gross-Umstadt, Germany), a three-line degasser (Jasco DG-1580-53), a ternary gradient unit (Jasco LG-1580-02) and an autosampler (Jasco AS-1550). A triple quadrupole mass spectrometer PE Sciex API 3000 equipped with a turbo ion spray operating in negative ionization mode was used for detection (Applied Biosystems, Darmstadt, Germany). High purity nitrogen for the mass spectrometer was produced by a nitrogen generator (Parker, Kaarst, Germany).

2.3. LC–MS/MS conditions for microdialysis samples

For the chromatographic separation a Synergi Hydro-RP column and precolumn were used (150 mm × 2 mm i.d., 4 μm particle size and 80 Å pore size from Phenomenex, Aschaffenburg, Germany).

In order to achieve short run times a linear gradient was employed at a flow rate of 0.5 ml/min mobile phase. Sample solvent was solvent C. Directly after injection of the sample (0 min) the gradient started from 100% solvent A to 100% solvent B within 1 min and then was held for 1 min at 100% solvent B (total 2.0 min). Within 0.3 min the mobile phase shifted back to 100% solvent A (total 2.3 min) and was held for another 3.7 min to equilibrate the column for the next sample. Total run time was 6 min. Injection volume of samples was 35 μl. Retention times of PGE_2 and PGD_2 were 2.51 ± 0.01 min (mean ± S.D., $n = 84$) and 2.76 ± 0.01 min (mean ± S.D., $n = 84$), respectively.

The mass spectrometer was operated in the negative ion mode with an electrospray voltage of −3400 V at 550 °C and was supplied by an auxiliary gas flow of 8.0 l/min. Nebulizer gas was set at 1.58 l/min and curtain gas at 1.25 l/min. Collision gas thickness was 2.28×10^{15} molecules/cm^2.

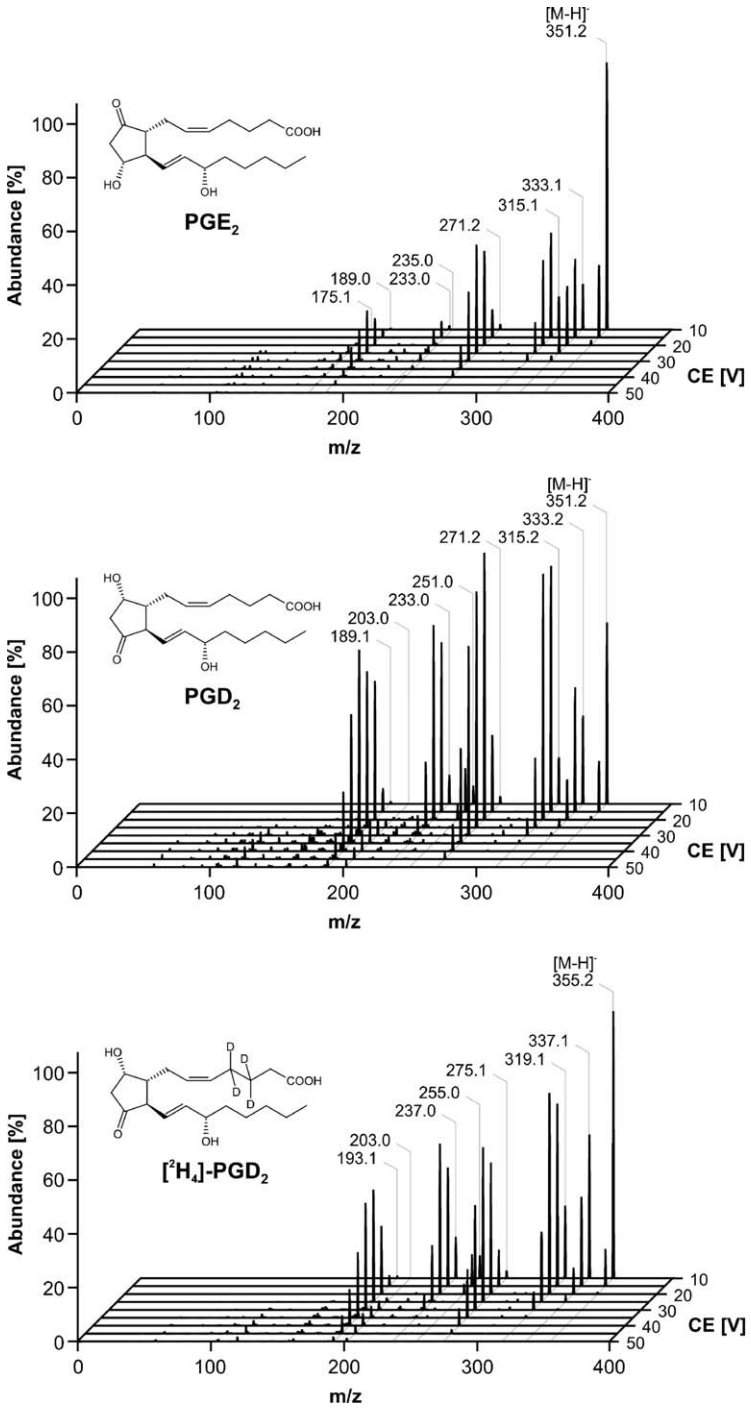
Multiple reaction monitoring (MRM) was used for quantification. The mass transitions used were m/z 351.2 → m/z 271.2 for PGE_2 and PGD_2 (collision energy −24 V) and m/z 355.2 → m/z 237.0 for the internal standard [$^2H_4$]-PGD_2 (collision energy −24 V) all with a dwell time of 200 ms.

All quadrupoles were working at unit resolution. Quantitation was performed with Analyst Software V1.1 (Applied Biosystems, Darmstadt, Germany) using the internal standard method (isotope-dilution mass spectrometry). Ratios of analyte peak area and internal standard peak area (y-axis) were plotted against concentration (x-axis) and calibration curves for each prostaglandin were calculated by least square regression with 1/concentration^2 weighting.

2.4. Standard preparation

A stock solution with 10,000 ng/ml of PGE_2 and PGD_2, respectively, was prepared in methanol. The stock solution was further diluted with methanol to obtain working standards with the concentration range of 87.5–3500 pg/ml. Working standards were prepared once a month. The concentration of the [$^2H_4$]-PGD_2 solution was 10 ng/ml in methanol. All solutions were stored at −80 °C.

Samples for standard curves and quality controls were prepared with 70 μl ACSF, 20 μl working standards (87.5–3500 pg/ml) and 20 μl internal standard [$^2H_4$]-PGD_2 (10 ng/ml) to obtain calibration standards from 25 to 1000 pg/ml and with a final concentration of [$^2H_4$]-PGD_2 of 4 ng/ml. Microdialysis samples of rats were prepared similarly and measured against a freshly prepared calibration curve, according to FDA recommendations [10].



PGE_2
$C_{20}H_{32}O_5$
Molecular mass 352.5 amu
Monoisotopic mass 352.2 amu

[M-H]⁻		Fragment	DP	FP	CE	CXP
351.2	→	175.1	-45	-185	-28	-3
351.2	→	189.0	-45	-185	-25	-9
351.2	→	233.0	-45	-185	-18	-5
351.2	→	234.9	-45	-185	-28	-5
351.2	→	271.2	-45	-185	-24	-2
351.2	→	315.1	-45	-185	-17	-1
351.2	→	333.1	-45	-185	-16	-5

PGD_2
$C_{20}H_{32}O_5$
Molecular mass 352.5 amu
Monoisotopic mass 352.2 amu

[M-H]⁻		Fragment	DP	FP	CE	CXP
351.3	→	189.1	-51	-170	-26	-11
351.3	→	203.0	-51	-170	-34	-15
351.3	→	233.0	-51	-170	-18	-7
351.3	→	251.0	-51	-170	-14	-7
351.3	→	271.2	-51	-170	-24	-7
351.3	→	315.2	-51	-170	-18	-3
351.3	→	333.2	-51	-170	-12	-9

$[^2H_4]$-PGD_2
$C_{20}H_{28}D_4O_5$
Molecular mass 356.5 amu
Monoisotopic mass 356.3 amu

[M-H]⁻		Fragment	DP	FP	CE	CXP
355.2	→	193.1	-22	-100	-26	-11
355.2	→	203.0	-22	-100	-38	-11
355.2	→	237.0	-22	-100	-17	-10
355.2	→	254.9	-22	-100	-18	-5
355.2	→	275.1	-22	-100	-24	-18
355.2	→	319.1	-22	-100	-17	-14
355.2	→	337.1	-22	-100	-12	-4

Fig. 1. Mass spectra of PGE_2, PGD_2 and $[^2H_4]$-PGD_2 by using product ion scans in the negative ionization mode and several collision energies. The inserted tables present the optimized multiple reaction monitoring (MRM) conditions for the mass transitions of PGE_2, PGD_2 and $[^2H_4]$-PGD_2 (DP: declustering potential, FP: focusing potential, CE: collision energy, CXP: cell exit potential). Product ion scans were obtained by infusion of 1000 ng/ml methanolic solutions of PGE_2, PGD_2 and $[^2H_4]$-PGD_2 (10 μl/min). Twenty-five product ion scans at each collision energy (10–50 V, step 5 V) were summed up.

2.5. Sample extraction

Prostaglandins were extracted with liquid–liquid extraction. Therefore, 1200 μl ethyl acetate was added to the samples without adjusting pH and vortexed for 30 s. After 2 min of centrifugation at 10,000 × *g*, the ethyl acetate phase was taken off and the solvent was removed at a temperature of 45 °C under a gentle stream of nitrogen. The residue was reconstituted with 50 μl of solvent C and centrifuged for 2 min at 10,000 × *g*. The solution was then transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC–MS/MS system.

2.6. Microdialysis

Male Sprague–Dawley rats (250–300 g, Charles River, Sulzfeld, Germany) were used for the microdialysis experi-

ments. Animals had free access to food and water. They were maintained in climate- and light-controlled rooms (24 ± 0.5 °C, 12/12 h dark/light cycle). The ethics guidelines for investigations in conscious animals were obeyed and the local Ethics Committee for Animal Research approved the experiments.

Animals were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg) for induction of anesthesia and intraoperative analgesia. During surgery, they were deeply anesthetized by a constant flow of isoflurane (1–1.5 vol.%). A controlled heating pad (CMA, Stockholm, Sweden) kept the core temperature at 37 °C. An incision was made over the spinal cord, and muscle tissue was cleared away from vertebrae T13 and L1. The animal was then mounted into a stereotaxic frame (David, Kopf Instruments) stabilizing the vertebral column without interference from rib cage movements. Small holes were drilled (with a diamond drill) through the lateral surface of vertebra T13 at the level of the dorsal horn. A dialysis probe constructed from a polyacrylonitrile hollow fiber (AN69; 0.2 mm i.d.; molecular mass cutoff ∼40 kDa; Hospal, Nürnberg, Germany) was placed through the holes, passing transversely through the dorsal horns of the spinal cord as described previously [11]. The dialysis membrane was meticulously covered with epoxy glue except for the region that was placed through the spinal cord. The ends of the dialysis tube were connected to polyethylene tubes, which were passed subcutaneously to the neck and externalized. At the end of surgery, animals received 50 mg/kg ketamine i.p. for postoperative analgesia and were allowed to recover in individual cages.

After a 24 h recovery period, the polyethylene tubes of the rat were attached to a microdialysis pump (CMA 100, CMA, Stockholm, Sweden) and perfused with ACSF at a flow rate of 5 μl/min. Animals were placed in a freely moving system (CMA, Stockholm, Sweden), and samples were collected in glass vials in 15 min intervals (75 μl sample volume). Collection of the samples started 2 h before formalin injection to ascertain prostaglandin baseline concentrations and stopped after 5 h following injection of 15 μl formalin (5 vol.%) into one hindpaw. At completion, the correct placement of the microdialysis catheter (dorsal horn, L4) was confirmed by microscopic inspection.

Microdialysis samples, standard calibration samples and quality controls were always extracted the same day. Immediately before LC–MS/MS-analysis 50 μl of solvent C were added, vortexed, centrifuged and transferred into glass vials. LC–MS/MS-analysis was performed within 3 days after collection of microdialysates.

3. Results and discussion

3.1. Optimization of the LC–MS/MS conditions

Due to the fact, that in a preliminary study only PGE_2 and PGD_2 were detected in spinal cord microdialysates the method was only optimized for PGE_2 and PGD_2. To assess the optimal parameters of the mass spectrometer methanolic tuning solutions of 1000 ng/ml of the two prostaglandins were infused into the mass spectrometer with an infusion rate between 10 and 50 μl/min. Prostaglandins showed molecular peaks and significant fragments in the negative ion mode (Fig. 1). Substance specific voltages for declustering (DP), focusing (FP), entrance (EP), cell entrance (CE) and cell exit potential (CXP) were roughly optimized with the ramping function of the Analyst software. The resulting voltages were further optimized by hand.

As shown in Fig. 1 the mass spectra of PGE_2 and PGD_2 at different collision energies are almost equal and differ only in low abundant product ions of *m*/*z* 175, *m*/*z* 203, *m*/*z* 235 and *m*/*z* 251. Margalit et al. [12] used product ions of *m*/*z* 175 to determine PGE_2 and *m*/*z* 233 to monitor PGD_2 concentrations, but did not use an HPLC-column to separate both prostaglandins from each other. Both product ions (*m*/*z* 175 and *m*/*z* 233) are not selective for the respective prostaglandin and therefore the

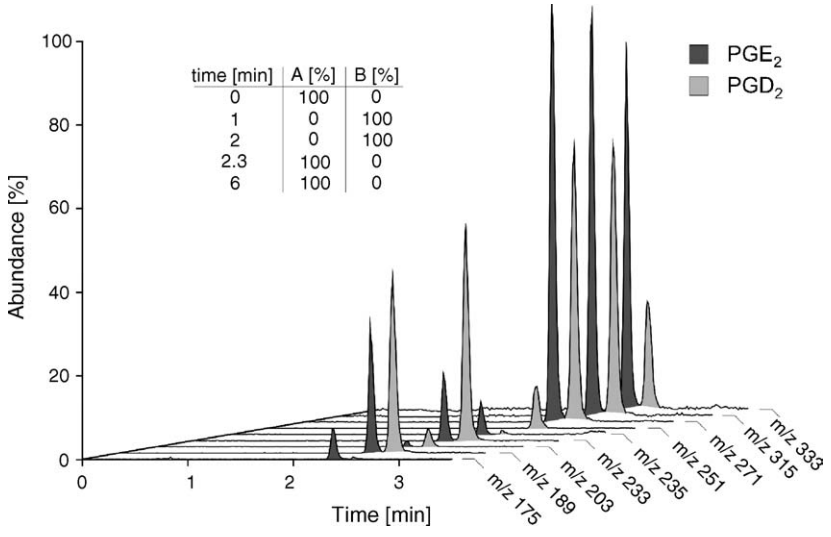


Fig. 2. Representative chromatograms of 2.5 ng/ml PGE_2 and 2.5 ng/ml PGD_2 standard sample using several mass transitions. A gradient run was used to obtain chromatograms (0 min: A 100%, B 0%; 1 min: A 0%, B 100%; 2 min: A 0%, B 100%; 2.3 min: A 100%, B 0%; 6 min: A 100% B 0%). Mobile phase A consisted of acetonitrile/water (40:60, v/v, pH 6.8), mobile phase B was methanol and sample solvent was methanol/water/formic acid (25:75:0.1, v/v, pH 2.8). Flowrate was 0.5 ml/min and injection volume was 35 μl.

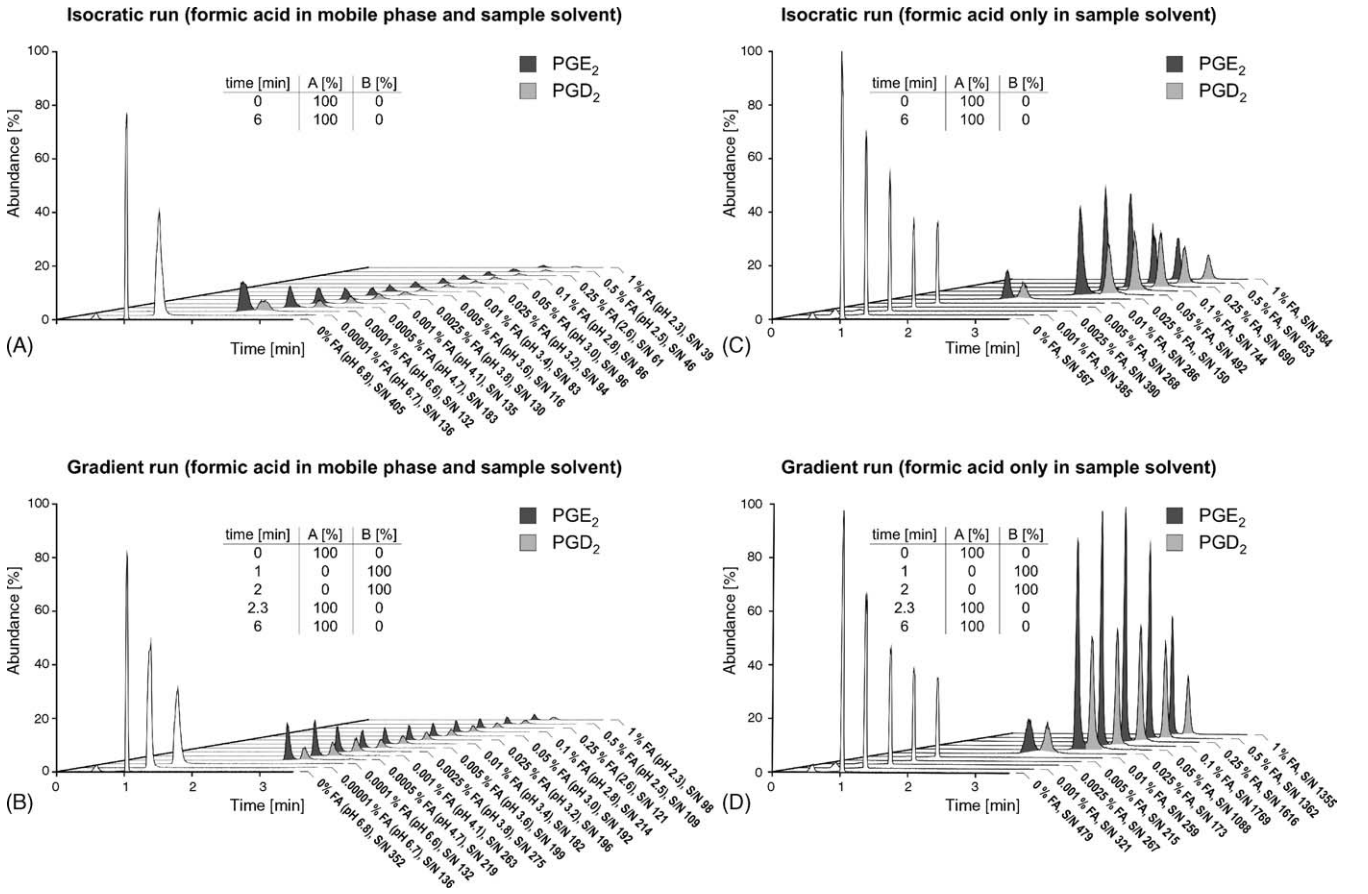


Fig. 3. Comparison of isocratic and gradient run with and without formic acid (FA) addition to the mobile phase. Chromatograms A and B were obtained using various concentrations of formic acid in mobile phase and sample solvent. The resulting pH refer to mobile phase A (acetonitrile/water/formic acid—40:60:*z*-axis, v/v) after formic acid addition. Concentration of PGE_2 and PGD_2 was 2.5 ng/ml in methanol/water/formic acid (25:75:*z*-axis, v/v). Chromatograms C and D were obtained using formic acid only in the sample solvent. Acetonitrile/water (40:60, v/v, pH 6.8) was used as mobile phase A and methanol as mobile phase B. Concentration of PGE_2 and PGD_2 was 2.5 ng/ml in methanol/water/formic acid, (25:75:*z*-axis, v/v). Chromatograms A and C were obtained under isocratic conditions (0 min: A 100%, B 0%; 6 min: A 100% B 0%), chromatograms B and D under gradient conditions (0 min: A 100%, B 0%; 1 min: A 0%, B 100%; 2 min: A 0%, B 100%; 2.3 min: A 100%, B 0%; 6 min: A 100% B 0%). Flowrate in all runs was 0.5 ml/min and injection volume was 35 μl. Signal-to-noise ratios (S/N) were calculated only for PGE_2.

assay resulted in interferences of ≥10%. Among the product ions observed only the ion at *m*/*z* 251 was specific for PGD_2 (Fig. 2). Quantification by using low abundant product ions (*m*/*z* 175 and *m*/*z* 251) would result in only 10–20% sensitivity as compared to *m*/*z* 271. Thus, PGE_2 and PGD_2 had been separated by liquid chromatography before mass spectrometric detection using product ion *m*/*z* 271 to obtain maximum sensitivity.

The best signal intensity was obtained with methanolic mobile phases. However, with mobile phases containing methanol, water and different modifiers such as ammonium acetate, ammonium hydroxide or formic acid a separation between PGE_2 and PGD_2 could not be achieved. Only mobile phases containing acetonitrile, water and formic acid as modifier resulted in a suitable separation of both prostaglandins on most reversed phase columns. Formic acid leads to decreased signal intensities in the negative ion mode where the prostaglandins are detected (Fig. 3). Thus, LC-conditions were not optimal for the mass spectrometer. The column with the best signal to noise ratio was the Synergi Hydro-RP (Phenomenex, Aschaffenburg, Germany).

Flow injection analysis (FIA) of a 5 ng/ml solution of PGE_2 and PGD_2, respectively, in solvent D was used to optimize other instrument parameters such as nebulizing gas (NEB), curtain gas (CUR), collision-activated dissociation (CAD) gas, electrospray voltage (IS), temperature, orientation of the electrospray needle and auxiliary gas flow.

Solvent D as mobile phase and sample solvent was well suited to separate PGE_2 and PGD_2, but the LLOQ of approximately 200 pg/ml was not sensitive enough to measure spinal cord microdialysis samples of rats because according to published data concentrations are in the range of 50–150 pg/ml PGE_2 [3,6,13].

When omitting the formic acid from the mobile phase (solvent A), but leaving it in the sample solvent in a concentration of 0.1% (solvent E), a separation of PGE_2 and PGD_2 was still possible, but a better signal-to-noise ratio was observed (Fig. 3A as compared to Fig. 3C). Solvent C as sample solvent showed the best sensitivity.

When using an isocratic run with solvent A as mobile phase and solvent C as sample solvent a run time of 11 min was nec-

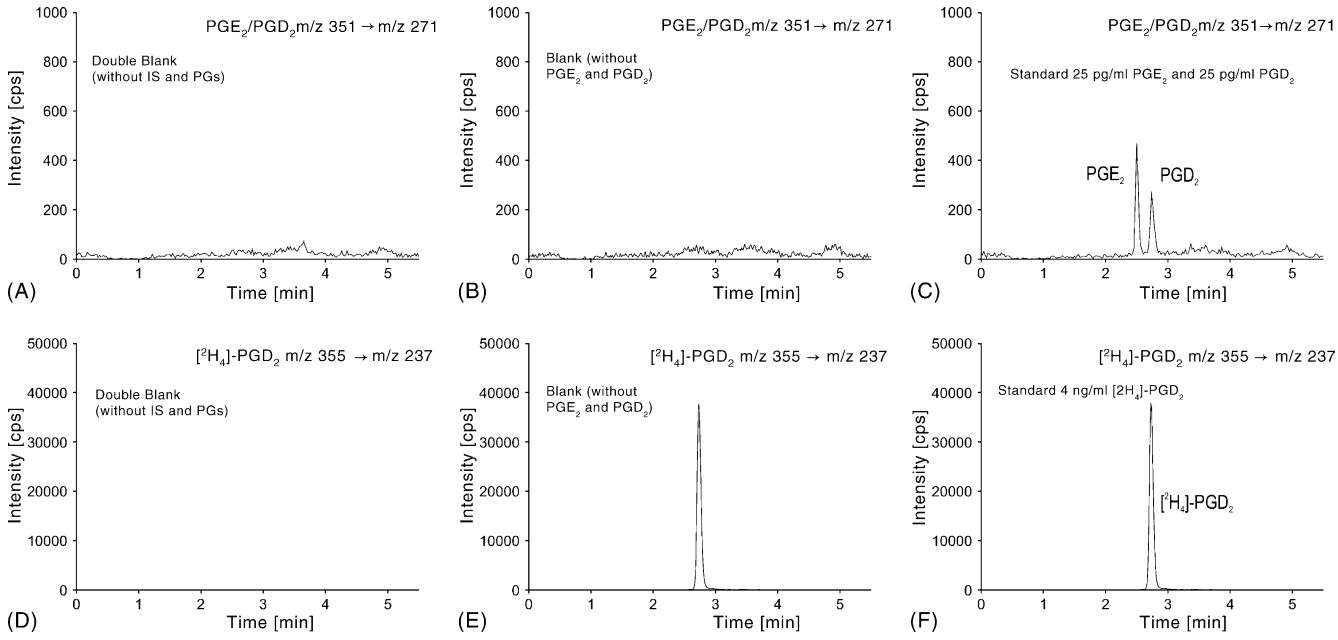


Fig. 4. Representative chromatograms of extracted standard samples using gradient elution. Mobile phase A consisted of acetonitrile/water (40:60, v/v, pH 6.8), mobile phase B was methanol and sample solvent was methanol/water/formic acid (25:75:0.1, v/v, pH 2.8). Chromatogram A represents an extracted nonspiked ACSF solution (double blank) obtained in MRM mode at transition m/z 351 → 271 for PGE_2 and PGD_2. The corresponding transition at m/z 355 → 237 for the internal standard $[^2H_4]$-PGD_2 is shown in chromatogram D. Chromatograms B and E (blank) represent an extracted ACSF solution with added concentrations of 10 ng/ml internal standard $[^2H_4]$-PGD_2 (final concentration 4 ng/ml). An extracted ACSF solution with added concentrations of 25 pg/ml PGE_2 (final concentration 35 pg/ml), 25 pg/ml PGD_2 (final concentration 35 pg/ml) and 10 ng/ml $[^2H_4]$-PGD_2 (final concentration 4 ng/ml) is shown in chromatograms C and F which represents the LLOQ of PGE_2.

essary to elute several other eicosanoids and the most lipophilic substance, arachidonic acid, from the Synergi Hydro-RP column. To reduce the run time a gradient was employed. As second part of the gradient methanol was selected because of its better sensitivity for prostaglandins as compared to acetonitrile. Gradient elution showed a two-fold gain in sensitivity as compared to isocratic run (Fig. 3).

Separation of PGE_2 and PGD_2 was performed on a Synergi Hydro-RP column within 6 min using gradient elution with acetonitrile/water (40:60, v/v, pH 6.8) as mobile phase A and methanol as mobile phase B. Flowrate was 500 μl/min and injection volume 35 μl. As sample solvent methanol/water/formic acid (25:75:0.1, v/v, pH 2.8) was used.

3.2. Development of the extraction procedure

The vehicle of spinal cord microdialysis samples, ACSF, contains high salt concentrations, which show a high ion suppression effect within the mass spectrometer. With increasing analysis time, salt accumulates in the ESI-chamber and leads to further decrease of signals, making it difficult to measure an acceptable number of samples in a row. To prevent the mass spectrometer from salt impurities and to develop a robust high throughput method we decided to extract the prostaglandins from microdialysis samples.

Although several extraction procedures for prostaglandins such as SPE and liquid–liquid extraction were previously described they were tested for an optimal signal-to-noise ratio [14–16]. Liquid–liquid extraction by using ethyl acetate showed a better signal-to-noise ratio at similar recovery and a higher sample throughput than various SPEs. Acidification of the samples prior ethyl acetate extraction was not necessary. No difference of recovery was observed by using triplicate extraction with 400 μl or one extraction with 1200 μl of ethyl acetate.

Since higher sample throughput than SPE and clean extracts were produced with liquid–liquid extraction, 70 μl of microdialysis samples were extracted with 1200 μl of ethyl acetate.

3.3. Method validation

Since it is not possible to collect prostaglandin free microdialysates, all samples for method validation were prepared with the same ACSF solution as used in animal experiments.

Isotopic purity of $[^2H_4]$-PGD_2 was 99.99% and no PGD_2-background at a final concentration of 4 ng/ml was observed (Fig. 4). Although $[^2H_4]$-PGE_2 may also be a useful internal standard, isotopic purity of available $[^2H_4]$-PGE_2 was only 99.0% and thus resulted in PGE_2-background signals, when used as internal standard at a final concentration of 4 ng/ml (data not shown). Therefore, only $[^2H_4]$-PGD_2 was used as internal standard. Since product ion m/z 275 of $[^2H_4]$-PGD_2 showed a high baseline, quantification was performed with product ion m/z 237, which resulted in a better assay accuracy as compared to product ion m/z 275.

Assay accuracy was calculated with six different standard series in the range from 25 to 1000 pg/ml. For calculation, ratios between peak areas of analyte (PGE_2 or PGD_2) and internal standard $[^2H_4]$-PGD_2 were used. Best values were

Table 1
Accuracy of PGE_2 and PGD_2

PGE_2				PGD_2			
Nominal concentration (pg/ml)	Accuracy ± S.D. (n = 6)		R.S.D. (%)	Nominal concentration (pg/ml)	Accuracy ± S.D. (n = 6)		R.S.D. (%)
	pg/ml	%			pg/ml	%	
Blank	No peaks	–	–	Blank	No peaks	–	–
25	25.1 ± 1.1	100.2 ± 4.3	4.3	25	28.4 ± 7.8	113.6 ± 31.2	27.4
30	30.2 ± 2.1	100.6 ± 7.0	7.0	30	29.9 ± 3.4	99.5 ± 11.2	11.3
40	38.4 ± 2.6	96.0 ± 6.6	6.9	40	41.4 ± 6.4	103.4 ± 15.9	15.7
50	50.7 ± 3.6	101.4 ± 7.2	7.1	50	49.6 ± 3.1	99.1 ± 6.2	6.3
60	62.2 ± 5.7	103.6 ± 9.5	9.2	60	61.6 ± 4.9	102.6 ± 8.1	7.9
80	78.2 ± 2.3	97.7 ± 2.9	3.0	80	79.4 ± 7.4	99.2 ± 9.2	9.3
100	100.1 ± 5.8	100.1 ± 5.8	5.8	100	98.3 ± 7.3	98.3 ± 7.3	7.4
200	194.4 ± 12.2	97.2 ± 6.1	6.3	200	196.0 ± 4.4	98.0 ± 2.2	2.2
300	305.4 ± 17.7	101.8 ± 5.9	5.8	300	313.8 ± 18.3	104.6 ± 6.1	5.8
600	604.8 ± 52.2	100.8 ± 8.7	8.7	600	585.0 ± 22.8	97.5 ± 3.8	3.9
1000	996.0 ± 32.0	99.6 ± 3.2	3.2	1000	1007.0 ± 16.0	100.7 ± 1.6	1.6
	Mean	99.9 ± 6.1	6.1		Mean	100.0 ± 5.6	5.6

Accuracy was determined using six different standard curves prepared in ACSF after extraction with ethyl acetate. No peaks of PGE_2 or PGD_2 were visible in unspiked ACSF samples.

obtained with weighted least square regression (weighting factor 1/concentration^2) which was used for all further calculations of the validation. Mean accuracy of the assay was found to be 99.9 ± 6.1% over the PGE_2 calibration range and 100 ± 5.7% over the PGD_2 calibration range. Detailed data are given in Table 1.

Intraday precision of the assay was determined using five concentrations (25, 50, 100, 300 and 1000 pg/ml), blank (with internal standard spiked matrix) and double blank samples (only matrix). Due to the large injection volume of 35 μl, only one injection per sample was possible and several reconstituted extracts of the same concentration had to be combined. Each sample was then analyzed 18 times in a row (Table 2). Intraday precision was repeated on four different days and values were used to calculate the interday precision (Table 2). LLOQ was defined as the concentration where standard deviation of accuracy did not exceed 15% and relative standard deviation (R.S.D.) of intra- and interday precision was less than 15%. LLOQ for PGE_2 and PGD_2 was found to be 25 pg/ml (1.2 pg on column) and 50 pg/ml (2.5 pg on column), respectively.

Extraction efficacy of prostaglandins was determined at four different concentrations (50, 100, 300 and 1000 pg/ml) whereby the recovery of the internal standard was determined only at the final concentration of 4 ng/ml. Relative recovery was calculated by comparing the mean peak areas of six extracted standards of one concentration with the mean peak areas of six

Table 2
Intra- and interday precision for PGE_2 and PGD_2 of extracted ACSF standard samples

Nominal concentration (pg/ml)	PGE_2		PGD_2	
	Mean (%) ± S.D. (n = 18)	Precision, R.S.D. (%)	Mean (%) ± S.D. (n = 18)	Precision, R.S.D. (%)
Intraday				
Blank	No peaks	–	No peaks	–
25	100.2 ± 14.0	13.9	104.8 ± 14.6	14.0
50	97.5 ± 6.7	6.8	100.6 ± 11.8	11.7
100	103.5 ± 7.7	7.4	106.6 ± 8.3	7.8
300	97.6 ± 5.5	5.7	96.9 ± 4.2	4.3
1000	100.4 ± 3.3	3.3	100.5 ± 4.0	4.0
Nominal concentration (pg/ml)	PGE_2		PGD_2	
	Mean (%) ± S.D. (n = 4 days)	Precision, R.S.D. (%)	Mean (%) ± S.D. (n = 4 days)	Precision, R.S.D. (%)
Interday				
Blank	No peaks	–	No peaks	–
25	99.6 ± 13.0	13.1	102.4 ± 17.3	17.0
50	100.2 ± 9.0	9.0	99.4 ± 9.0	9.1
100	100.7 ± 8.4	8.3	103.2 ± 9.4	9.1
300	99.5 ± 5.8	5.8	99.7 ± 7.0	7.0
1000	100.1 ± 4.6	4.6	99.9 ± 4.3	4.3

No peaks of PGE_2 or PGD_2 were visible in unspiked ACSF samples.

Table 3
Relative (as compared to spiked matrix samples) and absolute recoveries (as compared to spiked matrix free samples) for PGE_2, PGD_2 and $[^2H_4]$-PGD_2 of extracted ACSF standard samples

Nominal concentration (pg/ml)	PGE_2 Mean (%) ± S.D. ($n = 6$)	PGD_2 Mean (%) ± S.D. ($n = 6$)	$[^2H_4]$-PGD_2 Mean (%) ± S.D. ($n = 6$)
Relative recovery			
50	85.9 ± 6.3	80.3 ± 9.5	78.5 ± 2.9
100	82.5 ± 9.7	83.4 ± 13.5	86.0 ± 2.7
300	80.0 ± 2.2	80.4 ± 6.4	84.3 ± 2.7
1000	82.0 ± 1.1	77.2 ± 3.4	80.9 ± 2.3
Mean (%) ± S.D.	82.4 ± 4.8	80.3 ± 8.2	82.4 ± 2.6
Absolute recovery			
50	81.7 ± 8.1	75.2 ± 13.0	78.1 ± 2.7
100	81.8 ± 5.4	80.1 ± 5.5	83.5 ± 4.4
300	78.9 ± 4.1	78.1 ± 5.1	80.1 ± 2.5
1000	81.0 ± 1.6	73.7 ± 3.7	80.7 ± 2.6
Mean (%) ± S.D.	80.8 ± 4.8	76.8 ± ..6.9	80.6 ± 3.1

Final concentration of $[^2H_4]$-PGD_2 in each sample was 4 ng/ml.

extracted blank matrix samples which were reconstituted after evaporation of the ethyl acetate fraction with standards prepared in solvent C. Relative recovery was constant over the calibration range. Mean relative recovery was 82.4 ± 4.8% for PGE_2, 80.3 ± 8.2% for PGD_2 and 82.4 ± 2.6% for the internal standard $[^2H_4]$-PGD_2 (Table 3). On the other hand, absolute recovery was ascertained by comparing mean peak areas of extracted samples with matrix free solvent-standards in solvent C. Absolute recovery was constant over the calibration range and similar to relative recovery. Mean absolute recovery was 80.8 ± 4.8% for PGE_2, 76.8 ± 6.9% for PGD_2 and 80.6 ± 3.1% for the internal standard $[^2H_4]$-PGD_2 (Table 3).

Matrix and suppression effects were assessed with 36 extracted blank matrix samples, which were reconstituted with a 200 pg/ml standard in solvent C. The mean peak areas of all samples were compared with the mean peak areas of 36 matrix free 200 pg/ml standards in solvent C. Only a slight ion suppression effect of 7 ± 5.1% for PGE_2, 7.9 ± 8% for PGD_2 and 6 ± 5.9% for $[^2H_4]$-PGD_2 was observed. Since $[^2H_4]$-PGD_2 behave similar to both prostaglandins, PGE_2 and PGD_2 showed no matrix effect.

3.4. Stability of prostaglandins

After collection of real spinal cord microdialysates the samples were immediately extracted, stored at −80 °C and measured within 3 days. Each freeze/thaw stability, stability under storage conditions and short-term stability of PGE_2 and PGD_2 was determined with eight standard samples of two different concentrations (50 and 1000 pg/ml). The final concentration of the internal standard $[^2H_4]$-PGD_2 in the stability tests was always 4 ng/ml. Due to the low volume of microdialysis samples, no stability of real samples was determined. Samples for freeze/thaw stability were extracted and immediately frozen at −80 °C without solvent. After 24 h, the samples were stored for 30 min at room temperature and refrozen at −80 °C. After the third thaw-

Table 4
Recovery of extracted ACSF standard samples after performing storage-, short-term- and freeze/thaw-stability for PGE_2 and PGD_2 and stability of stock solutions of PGE_2, PGD_2 and $[^2H_4]$-PGD_2

Nominal concentration (pg/ml)	PGE_2 Mean (%) ± S.D. ($n = 8$)	PGD_2 Mean (%) ± S.D. ($n = 8$)
Storage-stability		
50	103.3 ± 4.5	104.5 ± 4.8
1000	105.7 ± 2.9	104.4 ± 1.9
Short-term-stability		
50	95.9 ± 3.9	90.6 ± 7.8
1000	99.3 ± 3.7	103.5 ± 3.5
Freeze/thaw-stability		
50	106.9 ± 7.9	101.9 ± 6.3
1000	105.8 ± 2.2	104.1 ± 3.6

Nominal concentration (ng/ml)	PGE_2 Mean (%) ± S.D. ($n = 6$)	PGD_2 Mean (%) ± S.D. ($n = 6$)	$[^2H_4]$-PGD_2 Mean (%) ± S.D. ($n = 6$)
Stock-stability			
100	101.3 ± 2.0	103.2 ± 4.3	104.7 ± 4.1

ing cycle, samples were reconstituted with 50 μl solvent C and measured against a freshly prepared calibration curve (Table 4). Prostaglandins were stable under the freeze/thaw stability conditions.

Stability under the storage conditions of −80 °C was ascertained over a period of one week. Therefore, samples were extracted and immediately frozen at −80 °C. No difference was observed to freshly prepared calibration standards.

To assess short-term stability, samples were extracted, reconstituted with 50 μl solvent C and stored at room temperature. After 24 h, the samples were measured against a freshly prepared calibration curve (Table 4). Prostaglandins were stable under room temperature.

Six different stock solutions of each compound were freshly prepared and stored for 6 h at room temperature. PGD_2 and PGE_2 stock solutions contained 10,000 ng/ml in methanol and concentration of internal standard solution [$^2H_4$]-PGD_2 was 1000 ng/ml in methanol. To assess the stability of the stock solutions they were diluted to a concentration of 100 ng/ml with methanol/formic acid (100:0.4, v/v, pH 2.5) and measured against freshly prepared calibration curves, whereas 10 μl was injected into the LC–MS/MS-system (Table 4). Stock solutions stored at room temperature showed no stability problem.

During all stability determinations, no difference was observed to freshly prepared calibration standards.

3.5. Interferences in LC–MS/MS

Due to the huge number of similar eicosanoids interferences may occur within the assay. To evaluate possible interferences, various other eicosanoids of the same molecular weight were tested.

PGH_2 showed the same fragmentation pattern and the same retention time as compared to PGE_2. Signal intensity of PGH_2 is

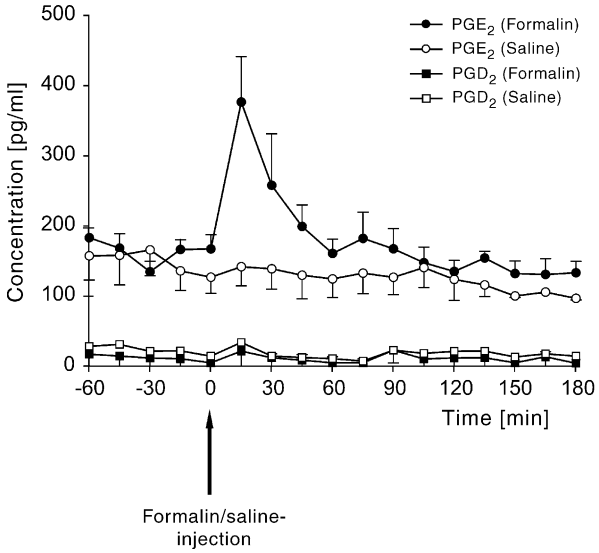


Fig. 5. Concentration of PGE_2 and PGD_2 in microdialysates from rats measured with LC–MS/MS. Data are presented as the mean ± standard error. Each group consisted of three rats.

~1000-fold weaker, and only very high concentrations of PGH_2 may affect PGE_2 determination. Furthermore, PGH_2 is rapidly converted by various prostaglandin synthases to the four major prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 and to thromboxane A_2 (TXA_2) and probably does not affect the assay at physiological concentrations.

An interference between PGD_2 and Δ^{12}-PGD_2 was observed. Δ^{12}-PGD_2 is an albumin catalyzed metabolite of PGD_2 itself, which is further metabolized to Δ^{12}-PGJ_2 and thiol conjugates. PGD_2 and Δ^{12}-PGD_2 showed nearly the same retention times, same fragmentation patterns and same signal intensities (100% interference). Another possible interference of PGE_2 may be

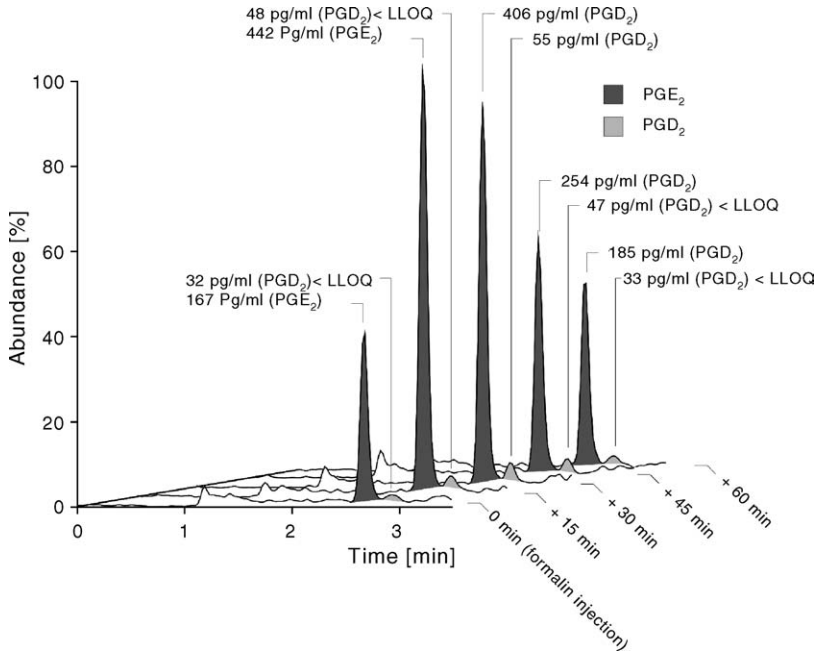


Fig. 6. LC–MS/MS chromatograms from microdialysate samples of a rat taken at various time points before and after injection of formalin (t = 0 min).

Δ^{12}-PGE_2. Interference of Δ^{12}-PGE_2, however, was not tested, because it was not available to us. No column tested was able to achieve an acceptable separation between these Δ^{12}-metabolites and the respective mother compounds in short run times.

Despite the fact, that presence of PGH_2, Δ^{12}-PGE_2, and Δ^{12}-PGD_2 may affect the described LC–MS/MS-assay, they do most likely not interfere with the analysis of microdialysis samples. First, low physiological concentrations of PGH_2 would produce a non detectable PGE_2 signal. Second, Δ^{12}-PGD_2 is an intermediate between PGD_2 and Δ^{12}-PGJ_2 and no other PGJ_2-derivatives of that pathway were observed in rat spinal cord microdialysates (data not shown). Thus, it is unlikely that Δ^{12}-PGD_2 is present in rat spinal cord microdialysates. Third, so far it is not reported that Δ^{12}-PGE_2 exists in vivo.

3.6. Application of the assay

To test the applicability of the method three rats were injected with 5 vol.% formalin (15 μl) and three others with 0.9 vol.% NaCl solution (15 μl). Spinal cord microdialysis was performed as described above. Fifteen minutes after injection of formalin into one hindpaw PGE_2 concentrations increased to a maximum of ∼380 pg/ml and decreased to baseline within 60 min (Figs. 5 and 6). Control rats showed no increase of PGE_2-levels after saline injection. Notably, concentrations of PGD_2 were rather low and were under the LLOQ (50 pg/ml). The PGE_2 concentrations measured in this study are in line with published results obtained with immunoassays [6].

4. Conclusion

To elucidate the role of PGD_2 in animal models of inflammation, a commercially available PGD_2-methoxime-immunoassays was used to determine PGD_2 in biological fluids, but the concentrations fluctuated unacceptably high and no stable baseline was achieved (data not shown). In addition, in case of microdialysates measurement of PGE_2 and PGD2 in the same sample by using immunoassays was not possible due to the low sample volume of <75 μl. The developed LC–MS/MS assay was able to separate PGE_2 and PGD_2 on a phenomenex Synergi Hydro-RP column using gradient elution with acetonitrile/water (40:60, v/v, pH 6.8) and methanol in 6 min after liquid–liquid extraction with ethyl acetate. As sample solvent methanol/water/formic acid (25:75:0.1, v/v, pH 2.8) was used. The LLOQ of the LC–MS/MS assay was found to be 25 pg/ml (1.2 pg on column) for PGE_2 and 50 pg/ml (2.5 pg on column) for PGD_2, respectively, and is lower than in other LC–MS [17,18] or LC–MS/MS [12] methods for PGE_2 and PGD_2 described so far.

The developed LC–MS/MS assay was able to measure PGE_2 and PGD_2 in the same microdialysis samples. Analysis of spinal cord microdialysates of rats showed similar concentrations of PGE_2 as published previously by using immunoassays [6], but concentrations of PGD_2 were under the limit of quantification. To definitely show whether or not PGD_2 is also involved in spinal nociceptive processing more sensitive LC–MS/MS, GC–MS or GC–MS/MS instruments have to be used.

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